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Coordinate Regulation of Complex T Cell Populations Responding to Bacterial Infection

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Summary

Bacterial infections activate complex T cell populations that differ in size and antigen specificity. We used tetramerized MHC class I molecules complexed with *Listeria monocytogenes*–derived epitopes to characterize four distinct CD8⁺ T lymphocyte populations during bacterial infection. Surprisingly, T cell populations differing in antigen specificity expand, contract, and enter the T cell memory compartment synchronously. Because the four *L. monocytogenes* epitopes are presented with different efficiencies and have distinct stabilities in infected cells, our findings suggest that these factors do not determine in vivo T cell dynamics. While T cell activation requires antigen presentation, the timing and extent of T cell expansion appear to be regulated in a coordinated fashion independent of antigen quantity and stability.

Introduction

CD8⁺ cytolytic T lymphocytes (CTL) responding to viral or bacterial infection recognize pathogen-derived peptides presented on the cell surface by major hisocompatibility complex (MHC) class I molecules (Germain, 1994; Lehner and Cresswell, 1996). The large number of protein antigens expressed by many pathogens results in the presentation of a spectrum of epitopes that prime a hierarchy of CTL populations that differ in antigen specificity and magnitude (Most et al., 1996; Steven et al., 1996, 1997; Vitiello et al., 1996; Vijh and Pamer, 1997). Immunodominant epitopes elicit large T cell responses and subdominant epitopes elicit smaller responses (Sercarz et al., 1993). Viral and bacterial infections induce predictable T cell responses characterized by initial activation and expansion of antigen-specific effector T cell populations followed by contraction and establishment of a T cell memory compartment (Gessner et al., 1989; Doherty et al., 1992; Oehen et al., 1992; Ahmed and Gray, 1996). It is unclear, however, whether T cells specific for different antigens undergo activation, expansion, contraction, and transition into the memory population in unison. T cells specific for different epitopes may, for example, expand and contract with distinct kinetics, being influenced by their cognate epitopes' number or stability. Indeed, dominant epitopes have been shown to form more stable complexes with MHC molecules, while subdominant epitopes dissociate more readily (Sette et al., 1994; van den Burg et al., 1996; Levitsky et al., 1996). Furthermore, the efficiency of epitope generation, as determined by proteasome-mediated antigen degradation or transporter associated with antigen processing translocation of peptides into the endoplasmic reticulum, can also influence T cell responses (Gournier et al., 1995; Niedermann et al., 1995; Yellen-Shaw et al., 1997). Although substantial evidence supports a role for antigen processing efficiency and peptide/MHC stability in the determination of immunodominance, other studies suggest that the repertoire of available T cells determines the magnitude of the subsequent T lymphocyte response (Cole et al., 1994; Viner et al., 1995; Cao et al., 1996; Deng et al., 1997; Moudgil et al., 1997).

Listeria monocytogenes is a gram positive bacterium that elicits robust CTL responses that clear the pathogen from infected mice and provide long-lasting immunity (Harty and Bevan, 1992; Kaufmann, 1995). Large numbers of CTL responding to *L. monocytogenes* infection in BALB/c mice are specific for the immunodominant epitope $LLO_{91–99}$ presented by the H2-K^d MHC class I molecule (Pamer et al., 1997; Vijh and Pamer, 1997). Two other epitopes, $mpl_{84–92}$ and $p60_{449–457}$, elicit subdominant T cell responses, while a fourth epitope, $p60_{217–225}$, elicits an intermediate response (Busch et al., 1997; Vijh and Pamer, 1997). Remarkably, in the *L. monocytogenes* system the size of the T cell response does not correlate with the amount of antigen secreted by intracellular *L. monocytogenes* or the number of cognate epitopes presented on the infected cell surface (Pamer et al., 1997).

Investigations of the in vivo basis for immunodominance have been hampered by the inability to identify antigen-specific T lymphocytes directly. Recently, tetramerized MHC class I molecules folded with different epitopes were used to directly stain antigen-specific T cells (Altman et al., 1996). We applied this method to the *L. monocytogenes* system by tetramerizing soluble, murine H2-K^d molecules folded with each of the *L. monocytogenes*–derived epitopes as well as a nonantigenic self- peptide derived from the tyrosine kinase Jak1 (Janus kinase 1) (Falk et al., 1991). Our studies show that CTL populations that differ in antigen specificity expand, contract, and undergo phenotypic changes synchronously during the primary and recall response to *L. monocytogenes* infection. These findings suggest that T lymphocyte expansion and entry into the T cell memory compartment are not determined by antigen prevalence or the stability of cognate peptide/MHC complexes.

Results

Generation of H2-K^d Tetramers and Staining of *Listeria*-Specific CTL

Tetrameric MHC class I complexes folded with epitopes can be used to directly identify antigen-specific T cells among complex T cell populations (Altman et al., 1996). Because CTL of BALB/c mice respond to four different *L. monocytogenes*–derived peptides presented by H2-K^d (Busch et al., 1997; Pamer et al., 1997), we decided

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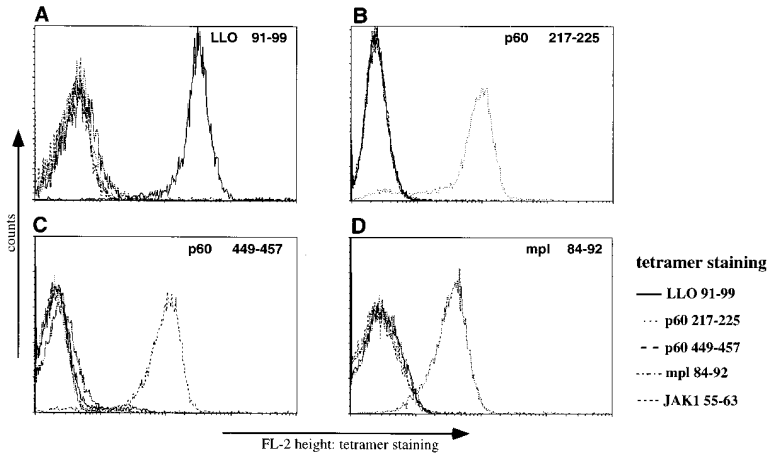

Figure 1. H2-K^d Tetramers Specifically Stain *L. monocytogenes*– Specific CTL Lines

CTL lines specific for each of the *L. monocytogenes*–derived epitopes were generated as described in Experimental Procedures. CTL were stained with CyChr–conjugated anti-CD8 and with each of the PE–conjugated H2-K^d tetramers. Histograms showing H2-K^d tetramer staining of CD8–gated CTL specific for $LLO_{91–99}$ (A), $p60_{217–225}$ (B), $p60_{449–457}$ (C), and $mpl_{84–92}$ (D) are shown.

to generate tetramers of H2-K^d complexed with each of these epitopes. The gene for H2-K^d was mutated to contain a biotinylation site (Schatz, 1993) at the C-terminus and expressed in *Escherichia coli*. H2-K^d and human β_2-microglobulin (β_2m) inclusion bodies were solubilized and refolded in the presence of each of the four *L. monocytogenes* epitopes ($LLO_{91–99}$, $p60_{217–225}$, $p60_{49–457}$, and $mpl_{84–92}$) as well as an H2-K^d–restricted, Jak1–derived self-peptide, following previously described procedures (Garboczi et al., 1996). Folded complexes, consisting of H2-K^d, β_2m, and antigenic peptide were enzymatically biotinylated with the biotin-protein ligase BirA, and tetramerized with phycoerythrin (PE)-labeled streptavidin.

The specificity of H2-K^d tetramers was determined with CTL lines that were generated by in vitro restimulation of immune, BALB/c splenocytes with each of the *L. monocytogenes*–derived epitopes. Previous studies have shown that CTL lines specific for $LLO_{91–99}$ and $p60_{217–225}$ have a complex T cell receptor (TCR) Vβ repertoire, while the responses to $p60_{449–457}$ and $mpl_{84–92}$ are more restricted (Busch and Pamer, 1998). H2-K^d/$LLO_{91–99}$ tetramers stained $LLO_{91–99}$–specific CTL (Figure 1A), but did not stain CTL specific for the three other epitopes (Figures 1B–1D). Similarly, H2-K^d tetramers formed with $p60_{217–225}$, $p60_{449–457}$, and $mpl_{84–92}$ stained only CTL lines with homologous specificity (Figure 1). Remarkably, tetramer staining identified the majority of antigen-specific CTL, although they consisted of complex populations with a multitude of different TCRs (Figures 1A and 1B). Additionally, tetramers also stained T cell lines specific for the subdominant epitopes $p60_{449–457}$ and $mpl_{84–92}$ (Figures 1C and 1D). These experiments therefore confirm the utility of tetramerized MHC class I molecules for the identification of antigen-specific T cell populations.

Tetramer Staining of CD8 Splenocytes following *Listeria* Infection

Purified, PE-labeled tetramers and fluorescein isothiocyanate (FITC)-conjugated antibodies for the activation markers CD62L and CD44 were used to stain CD8–enriched splenocytes derived from a BALB/c mouse immunized 7 days earlier with *L. monocytogenes*. CD62L (or L-selectin) expression is down-regulated after T cell activation, whereas CD44 expression is up-regulated. As shown in Figure 2A, 1.39% of all CD8 T cells stain with H2-K^d/$LLO_{91–99}$ tetramers and express low levels of CD62L. Similarly, 1.40% of CD8 T cells stain with $LLO_{91–99}$ tetramers and express high levels of CD44. Roughly 0.28% of CD8 T cells were stained with H2-K^d/$p60_{217–225}$ tetramers. Although the populations were small, H2-K^d/$p60_{449–457}$ and H2-K^d/$mpl_{84–92}$ tetramer-stained T cells were also identified within the CD62L^{low} population. Staining with H2-K^d/Jak1 tetramers did not stain any distinct populations of CD62L^{low} or CD44^{high} CD8 T cells and demonstrated the background staining attributable to nonspecific association of tetramers with T cells. The numbers of T cells stained with the K^d/tetramers containing *L. monocytogenes* epitopes correlated very closely with the T cell frequencies previously determined by interferon-γ enzyme-linked immunospot (Vijh and Pamer, 1997; Busch and Pamer, 1998).

Direct Ex Vivo Cytolysis by Tetramer-Purified CTL

To demonstrate that CD8$^+$ T cells stained with the various tetramers were antigen-specific, we sorted CD62L^{low}, $LLO_{91–99}$, and $p60_{217–225}$ tetramer-positive T cells and residual CD8$^+$ and CD62L^{low} T cells to assay them directly for antigen specificity (Figure 2B). This experiment demonstrates that T cells stained with $LLO_{91–99}$ or $p60_{217–225}$ tetramers are specific for their respective epitopes. Additionally, the cells from which $LLO_{91–99}$–specific T cells were sorted have lost all reactivity toward $LLO_{91–99}$ while maintaining reactivity toward $p60_{217–225}$. The same is true for staining of immune splenocytes with $p60_{217–225}$ tetramers (Figure 2B). These experiments indicate that tetramers stain essentially all antigen-specific CTL within the complex T cell population.

CTL Kinetics following Primary *Listeria* Infection

Immunodominance of $LLO_{91–99}$ and $p60_{217–225}$ does not correlate with their efficiency of antigen processing or presentation. In fact, $p60_{449–457}$ is generated more efficiently and is present in much larger amounts than the dominant epitopes (Sijts et al., 1996; Pamer et al., 1997). To determine whether T cells with different specificities expand with different kinetics, we characterized the expansion and contraction of CTL specific for the four H2-K^d–restricted epitopes at intervals following infection

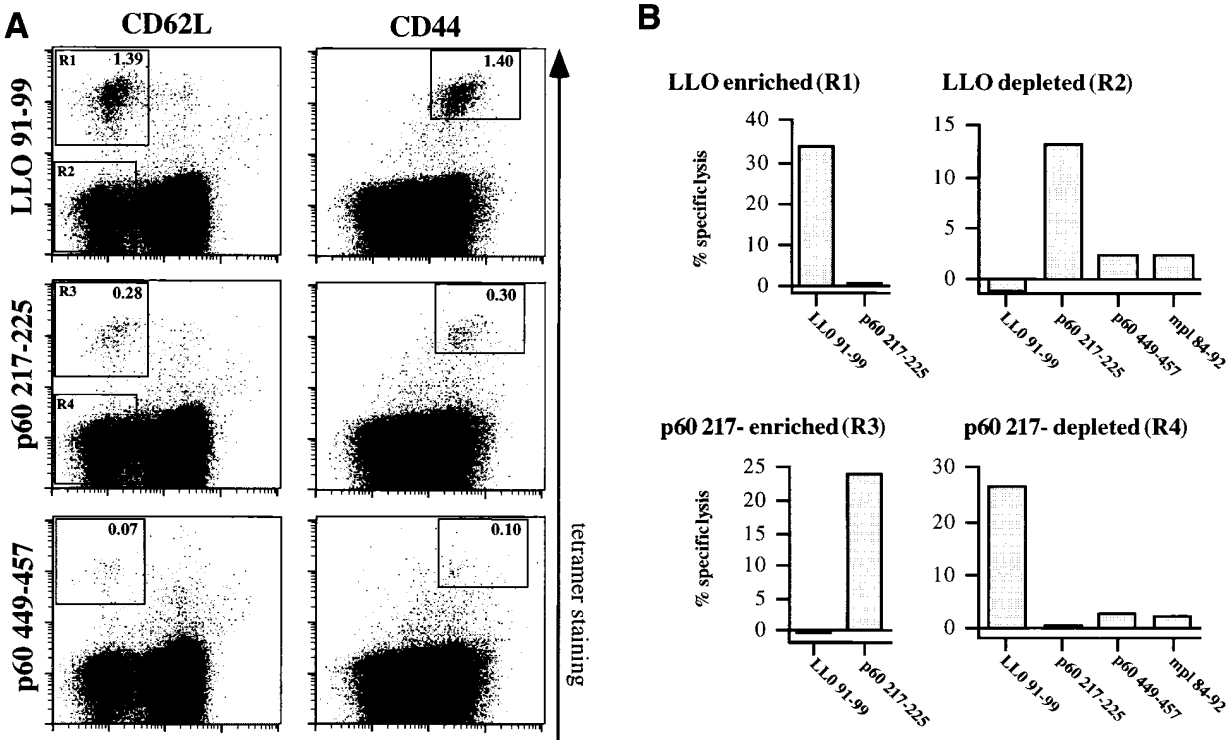

Figure 2. Direct Ex Vivo Detection of Epitope-Specific T Cell Populations Using Tetrameric MHC/Peptide Complexes

(A) Splenocytes were obtained 7 days after infection of a BALB/c ($H2^d$) mouse with a sublethal dose of *L. monocytogenes*. $CD8^+$ T cells were enriched by MACS (Miltenyi) and triple-stained with anti-CD8, anti-CD62L, or anti-CD44 and with PE–conjugated tetrameric H2-K^d/peptide complexes. Dot plots for 120,000 $CD8^+$ T cells are shown, with tetramer staining displayed on the y-axis and activation marker staining (CD62L and CD44) on the x-axis. Four log phases are shown.

(B) $CD8^+$ T cells were sorted from the region gates R1–R4 as indicated in (A) and were directly analyzed in CTL assays using peptide-coated, ^{51}Cr-labeled P815 ($H2^d$) target cells at effector-to-target cell ratios of approximately 0.3:1 (LLO– or $p60_{217–225}$-enriched) or 3:1 (LLO– or $p60_{217–225}$-depleted). The percent specific lysis is indicated.

with *L. monocytogenes*. Primary infection with 2000 *L. monocytogenes* is characterized by growth of bacteria in the spleen for the first 72 hr after infection followed by elimination of bacteria between days 3 and 7 following infection (data not shown). Infected spleens did not contain any epitope-specific T cells in the $CD62L^{low}$ population for the first 3 days after primary *L. monocytogenes* infection (Figure 3). On the fifth day of infection, however, a population of $LLO_{91–99}$–specific CTL is readily detected, as is a smaller $p60_{217–225}$–specific population (Figure 3). The peak response to all four epitopes occurs 7 days following infection, at which time small populations of $CD62L^{low}$ T cells are also detected with $p60_{449–457}$ and $mpl_{84–92}$ H2-K^d/tetramers. H2-K^d/tetramers presenting the Jak1 self-peptide did not stain activated $CD8^+$ T cells at any time during the course of the *L. monocytogenes*–specific T cell response (Figure 3), confirming the specificity of ex vivo staining with tetrameric H2-K^d complexes. Interestingly, the number of *L. monocytogenes*–specific T cells peaks on day 7 and then decreases similarly for each of the different epitope-specific subpopulations between days 9 and 14. However, between 14 and 35 days following infection the total number of antigen-specific T cells in immune spleens does not change significantly, as seen most readily with the dominant $LLO_{91–99}$–specific CTL population. However, a proportion of cells regain surface CD62L expression. Expression of CD62L by memory T cells has been shown previously in studies of the murine CTL response to HLA-Cw4 (Walker et al., 1995) and with TCR–transgenic T cells (Zimmermann et al., 1996; Pape et al., 1997a; Sprent, 1997). Antigen-specific CTL maintain high levels

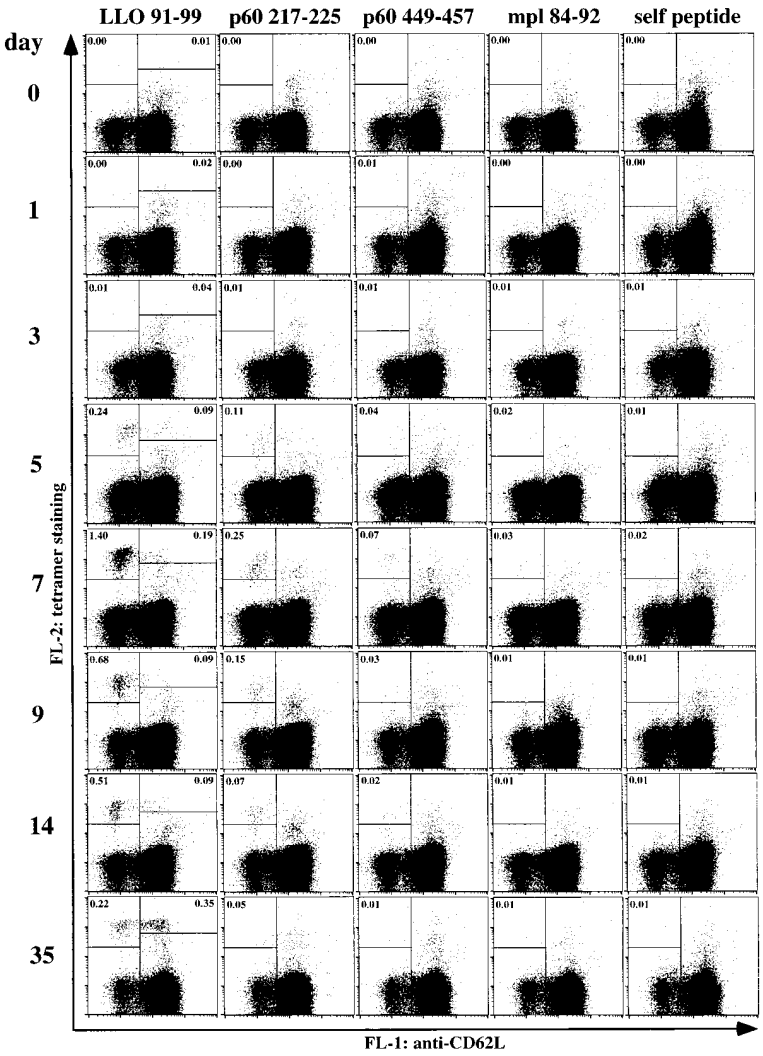

Figure 3. Kinetic Analysis of Epitope-Specific T Cell Populations during Primary Infection with *L. monocytogenes*

Age- and sex-matched BALB/c mice were infected with a sublethal dose of *L. monocytogenes.* Individual mice were sacrificed on the indicated days following infection, and $CD8^+$ T cells were enriched and stained for CD8, CD62L, and epitope-specific TCRs with each of the five PE–conjugated H2-K^d tetramers. The dot plots represent $CD8^+$ gated T cells stained with specific tetramers (y-axis) and CD62L (x-axis). Four log phases are shown.

of CD44 expression, however (D. H. B. and E. G. P., unpublished data), demonstrating that this surface molecule is a stable marker for "experienced" T lymphocytes, even in this complex T cell population.

Recall CTL Responses to *Listeria* Infection

We next investigated epitope-specific T cell populations during the recall response following *L. monocytogenes* reinfection. Although the bacterial inoculum is 50–fold greater during the recall response, only a few bacteria are detectable in the spleen 1 day after inoculation, and 72 hr after reinfection live bacteria are undetectable (data not shown). Epitope-specific memory T cells are already detectable by tetramer staining before reinfection, making it possible to follow and investigate the early stages of CTL activation and expansion. The first day after reinfection, the number of epitope-specific T cells in the spleen decreases (Figure 4). Although the reason for this is unknown, because *L. monocytogenes* causes a systemic infection it is possible that circulating, *L. monocytogenes*–specific CTL are targeted to nonsplenic sites of infection and thus decrease in number transiently in the spleen. There is a dramatic increase in the number of $CD62^{low}$ T cells specific for each of the epitopes 3 days following reinfection with *L. monocytogenes* (Figure 4). This increase continues until day 5 following reinfection, at which time a peak response to all four epitopes is seen. At day 5 during a recall response to *L. monocytogenes*, nearly 17% of all $CD8^+$ T lymphocytes and 40%–45% within the $CD8^+CD62L^{low}$ subpopulation in the spleen are specific for $LLO_{91–99}$. Between days 7 and 35 following reinfection there is a decrease in the number of all antigen-specific T cells detected by tetramer staining.

Synchronous Kinetics of *Listeria*-Specific CTL

A remarkable finding in this experiment is the synchronous expansion and contraction of CD8 T lymphocytes that are specific for different peptides (Figure 5). The number of CTL specific for each of the four epitopes is maximal 7 days following primary infection (Figures 5A and 5B) and then decreases 14 days following infection. The peak response to *L. monocytogenes* corresponds to the time that bacteria are cleared from the spleens

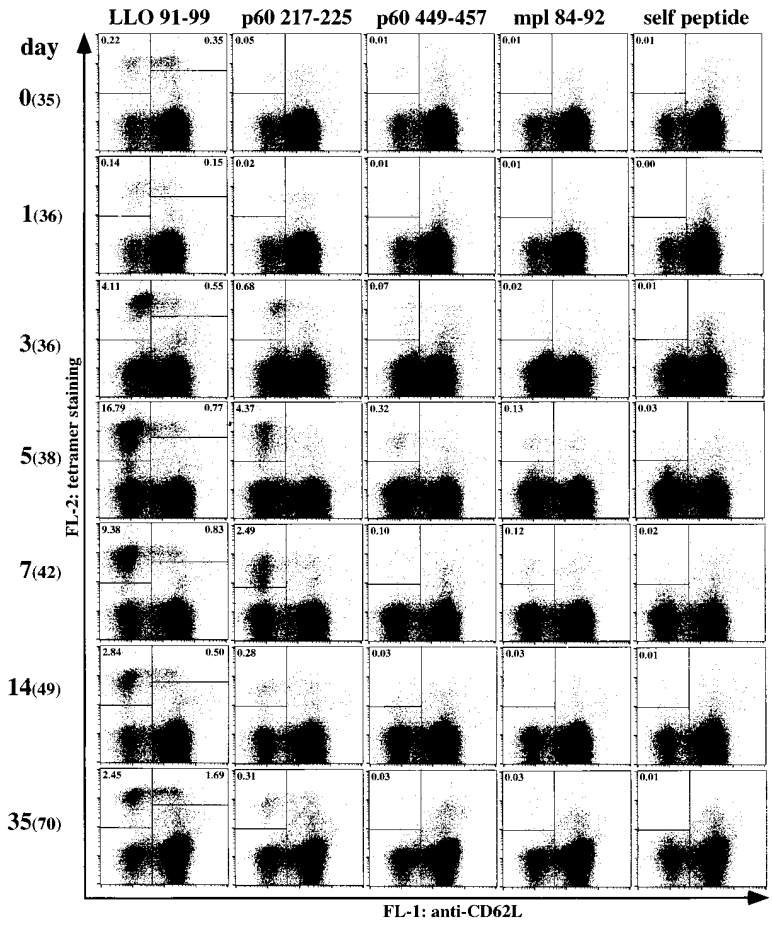

Figure 4. Kinetic Analysis of Epitope-Specific T Cell Populations during the Recall Response to *L. monocytogenes* Infection

Age- and sex-matched, immune BALB/c mice (infected 35 days previously with a sublethal dose of *L. monocytogenes*) were reinfected with 100,000 bacteria. $CD8^+$ T cells were enriched from spleens and stained and analyzed for CD62L and the five different H2-K^d tetramers as described in Figure 3.

of infected mice. Although the general profiles for the different antigen-specific T cell populations are similar, their extent of expansion are not identical. Thus, between days 5 and 7 following primary infection, $LLO_{91–99}$–specific cells undergo greater expansion than $p60_{217–225}$–specific cells. This finding warrants further investigation. The maximal recall response occurs 5 days after reinfection with *L. monocytogenes* (Figures 5C and 5D). Again, CTL specific for the dominant $LLO_{91–99}$ and $p60_{217–225}$ epitopes and the subdominant $p60_{449–457}$ and $mpl_{84–92}$ epitopes expand and contract synchronously. Remarkably, expansion of *L. monocytogenes*–specific CTL during the recall response occurs 48–72 hr after bacteria have been cleared from the spleens of infected mice.

Size and CD8 Expression of Responding CTL

Although the number of T cells specific for the *L. monocytogenes*–derived epitopes increases in the spleen, it could be argued that these increases reflect CTL recruitment to a principal site of infection rather than bona fide in situ expansion. Because activated, dividing T lymphocyte blasts are larger in size than resting T lymphocytes, we decided to examine this parameter (forward scatter [FSC]) on tetramer-stained T cells during the primary and recall response to *L. monocytogenes* infection (Figure 6A). As expected, the greatest changes in the FSC are detected on day 5 of the primary response and day 3 of the recall response. These times immediately precede the day when the largest T cell populations are detected, suggesting that T cell division plays a major role in the generation of larger numbers of antigen-specific T cells in the spleen. Comparison of FSC changes of T cells stained with tetramers again demonstrates the synchrony of different T cell populations regardless of their specificity for dominant or subdominant epitopes (Figures 6B–6E).

Activation of T lymphocytes has also been shown to result in the down-regulation of CD8 surface expression (Walker et al., 1995; Zimmermann et al., 1996; Viola et al., 1997). To confirm the synchrony of the different *L. monocytogenes*–specific CTL populations, we investigated this parameter during the primary and recall response to infection. The intensity of CD8 staining of $LLO_{91–99}$–specific CTL was significantly less than the intensity of $CD62L^{high}$, non–tetramer-stained T cells (Figure 6A). CD8 down-regulation was detectable during the primary immune response 7 days following infection and was maintained even 5 weeks after infection. Following reimmunization, H2-K^d tetramer–stained T cells up-regulated CD8 expression within 1 day, a shift that preceeded any increase in the number of antigen-specific T cells in the spleen. CD8 expression remained elevated 3 days following reinfection and then decreased again on the fifth day. Comparison of CD8 expression by CTL stained

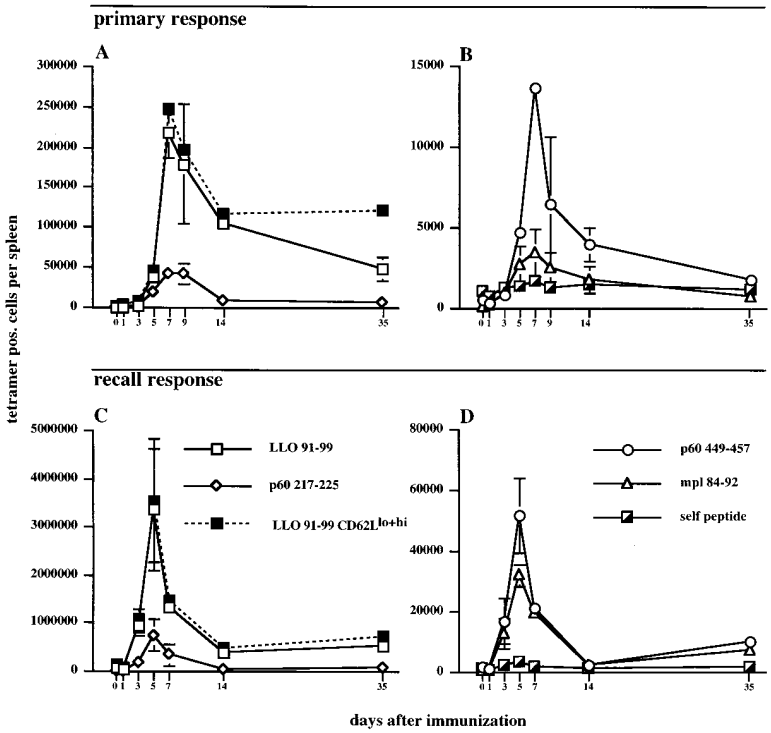

Figure 5. Primary and Recall Response to Four Different *Listeria*- Derived Epitopes Are Synchronous

The number of $CD8^+$, $CD62L^{low}$ T cells staining with each of the H2-K^d/epitope tetramers was quantified at each time point following primary infection and plotted as the absolute number of antigen-specific CD8 T cells per spleen. For $LLO_{91–99}$-specific CTL, the number of $CD62^{low}$ and $CD62L^{high}$ cells is also plotted. The mean value obtained from two mice and the standard deviation are shown for responses to primary infection (A and B) and following recall infection (C and D).

with the different tetramers showed that the timing of down-regulation following primary infection, transient up-regulation during the recall response, and subsequent down-regulation were similar among the different T cell populations (Figures 6B–6E).

Discussion

Complex pathogens elicit diverse T cell responses that have been categorized as dominant and subdominant. Why certain pathogen-derived peptides elicit large, vigorous T cell responses while others result in small, at times nearly undetectable, responses is uncertain. Our studies comparing diverse T cell populations responding to *L. monocytogenes* infection suggest the following: (1) dominant and subdominant T cell populations expand with similar kinetics; (2) diverse T cell populations responding to different antigens but the same infection undergo phenotypic changes synchronously; (3) the magnitude of the T cell response to different epitopes reflects the number of precursors available at the time of infection; and (4) while T cell activation is indisputably dependent upon antigen, the duration and extent of in vivo T cell expansion appear to be determined by factors other than antigen quantity or stability.

Previous studies of in vivo T cell expansion have used two methods. The first involves monitoring the expansion of highly uniform T cell populations that express a predominant TCR Vβ chain following immunization with antigens (McHeyzer-Williams and Davis, 1995; Walker et al., 1995; Maryanski et al., 1996). The responses characterized in these systems are directed at a single, highly dominant T cell epitope. The second method that has been used to investigate in vivo T cell response involves the adoptive transfer of TCR–transgenic, naive T cells into recipient mice (Kearney et al., 1994; Zimmermann et al., 1996). These studies have demonstrated impressive expansion of transferred cells upon antigenic stimulation and the requirements for costimulation for priming. Furthermore, they have demonstrated the negative impact of CTLA-4 action on in vivo T cell expansion (Kearney et al., 1995) and the positive impact of inflammatory cytokines (Pape et al., 1997b). Our study differs from these in that we are investigating a polyclonal, multispecific CTL response to infection by a complex pathogen. The CTL response is specific for four different epitopes derived from *L. monocytogenes*, and we know that CTL responding to $LLO_{91–99}$ and $p60_{217–225}$ express a diverse range of TCR Vβ chains (Busch and Pamer, 1998). Thus, the CTL populations identified in this study are highly diverse, a characteristic that likely typifies CTL responses occurring during most infectious diseases (Casanova et al., 1991; Cole et al., 1994; Cose et al., 1995).

Why are CTL responses to these different epitopes different in magnitude but kinetically similar? Although we have not yet been able to isolate or quantitate naive precursors in unimmunized mice, our data suggest that the ultimate size of an epitope-specific T cell response is determined by the naive precursor pool size. Certainly in the recall response the size of the recall CTL populations correlates with the size of the memory population. It is possible, however, that differences in the rate of expansion among the different T cell populations also contribute to the response hierarchy (note, for example, the differences between the ratio of $LLO_{91–99}$– and $p60_{217–225}$–specific T cells on days 5 and 7 after primary infection [Figure 5A]). Overall, however, when the response to all four epitopes is considered our results are most consistent with previous studies demonstrating the role of the TCR repertoire in determining the relative

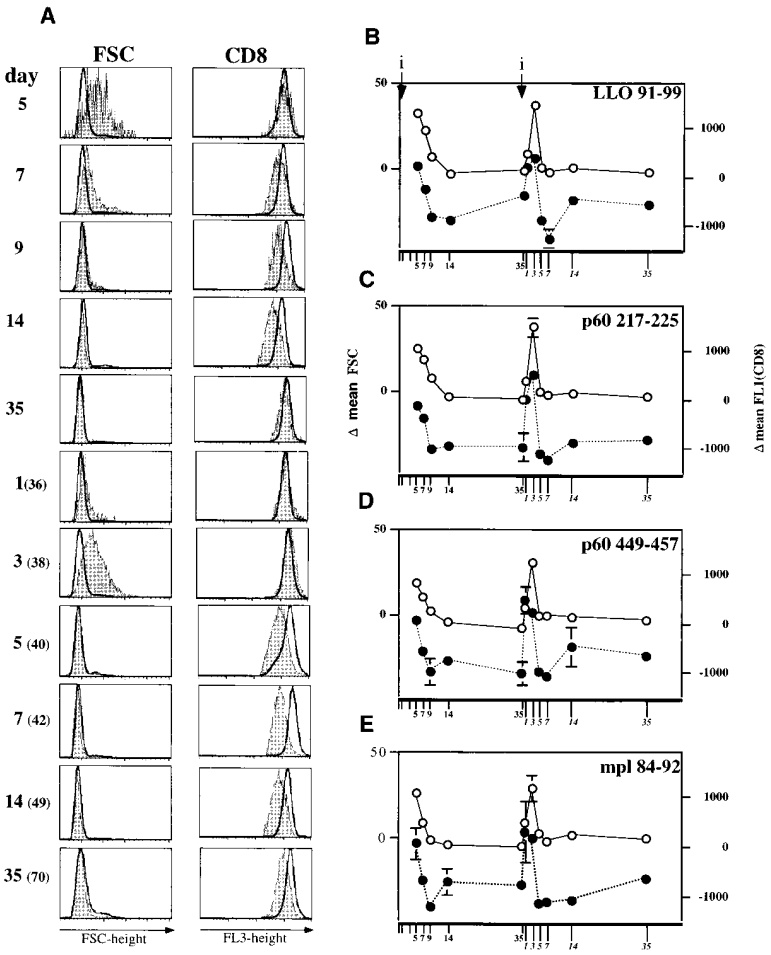

Figure 6. Phenotypic Shifts of Distinct T Cell Subpopulations Occur Synchronously during Primary and Recall Response to *L. monocytogenes* Infection

BALB/c mice were infected with *Listeria*, and epitope-specific $CD8^+$ T cells were analyzed on sequential days following infection. $CD8^+$-enriched splenocytes were stained for CD8, CD62L, and each of the four *L. monocytogenes* epitopes containing H2-K^d tetramers.

(A) Histograms demonstrate the FSC profiles of $CD8^+$, $CD62L^{low}$, and H2-K^d/$LLO_{91–99}$ tetramer-positive T cells (filled areas) and $CD8^+$, $CD62L^{high}$, and H2-K^d tetramer-negative T cells (black lines) on sequential days following primary and reinfection with *L. monocytogenes*. The level of CD8 expression is also indicated for the same T cell populations in the right column of histograms. FSC, linear scale (0–250); FL3, four log-phases.

(B–E) The differences in mean FSC (open circles, left y-axis) and mean CD8 expression (circles, right y-axis) between $CD8^+$, $CD62L^{low}$, tetramer-positive and $CD8^+$, $CD62L^{high}$, tetramer- negative T cell populations are plotted for each of the different epitope specificities at sequential timepoints during the primary and recall response to *L. monocytogenes* infection. The days following primary infection and reinfection (italic numbers) are indicated on the x-axis.

dominance of T cell responses to different epitopes (Cole et al., 1994; Viner et al., 1995; Cao et al., 1996; Deng et al., 1997; Moudgil et al., 1997).

The synchrony of the diverse, multispecific CTL response to *L. monocytogenes* infection is striking. In primary infection and especially following reinfection the major burst of CTL expansion occurs after viable *L. monocytogenes* have been eliminated. Expansion of CTL ceases long (>72 hr) after viable bacteria have been eliminated. Although peptide/MHC complexes are clearly required for CTL priming, the stimulus for prolonged CTL expansion is unknown. Do peptide-stimulated CTL undergo subsequent divisions independent of further peptide stimulation, or is their continued division promoted by the persistence of peptide/MHC complexes? Although our experiments do not definitively answer this question, several points argue in favor of the former possibility. First, the antigens from which the four *L. monocytogenes* epitopes are derived are expressed by *L. monocytogenes* in vastly different amounts (Villanueva et al., 1995). Second, the H2-K^d–associated epitopes are processed with different efficiencies and are present in infected macrophages in different amounts (Pamer, 1994; Villanueva et al., 1994; Sijts et al., 1996). Third, the *L. monocytogenes*–derived epitopes dissociate from H2-K^d at markedly different rates; $LLO_{91–99}$ and $p60_{217–225}$ form stable complexes with H2-K^d with half-lives exceeding 6 hr, while $p60_{449–457}$ and $mpl_{84–92}$ form unstable complexes, with half-lives of 1 hr (Sijts and Pamer, 1997). If antigen is driving in vivo expansion of CTL specific for the four different epitopes, the cessation of expansion could be explained by the eventual disappearance of H2-K^d/epitope complexes. This seems implausible, however, given the differences in antigen amount and stability. It seems much likelier that T cell expansion and the cessation of expansion are controlled by factors associated with the innate inflammatory response to infection (Pape et al., 1997b; Unanue, 1997).

Why do CTL that are specific for different epitopes stop expanding at the same time? Several scenarios are possible. Expression of an inhibitory receptor on activated CTL, such as CTLA-4, may be up-regulated on all *L. monocytogenes*–specific CTL synchronously regardless of their antigen specificity (Krummel and Allison, 1996; Walunas et al., 1996). The stimulus for up-regulation of such an inhibitory factor is unknown. Alternatively, the cytokine milieu surrounding CTL may change from one that supports T cell division to one that does not (Pape et al., 1997b). Given the substantial inflammatory response induced by *L. monocytogenes* and the induction of interleukin-12 (Hsieh et al., 1993),

it would not be surprising if T cell expansion were tied to the innate inflammatory response. Understanding the mechanisms that control T cell expansion during infection by complex pathogens will require further experiments. The results of these studies, however, may provide useful information for maximizing T cell responses in the case of vaccine development or minimizing T cell responses in autoimmune diseases.

Experimental Procedures

Mice, Cell Lines, and *Listeria* Infections

BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, ME). The P815 mastocytoma ($H2^d$) cell line was obtained from the American Type Culture Center (Rockville, MD) and maintained in RPMI 10% fetal calf serum. For primary infection, 2000 *L. monocytogenes* 10403S (obtained from Daniel Portnoy, Berkeley, CA) were injected into the tail vein of 8- to 10-week-old BALB/c mice, and splenocytes were harvested at different points during the course of infection. Reimmunization was performed by intrevenous injection of 100,000 *L. monocytogenes* 10403S 5 weeks after primary infection.

Generation of H2-K^d Tetramers

Tetrameric H2-K^d/peptide complexes were generated following the approach recently described by Altman et al. (1996). To obtain high yields of monomeric, biotinylated H2-K^d/peptide complexes, we modified refolding conditions and optimized the in vitro biotinylation. Full-length cDNA of H2-K^d heavy chain and human β_2m were kindly provided by Pamela Bjorkman, California Institute of Technology, CA. The extracellular domain of the H2-K^d molecule (truncated after amino acid 283) was fused to a specific biotinylation site (sequence #45, as reported by Schatz [1993]), using the following primers: 5′-GGCATATGGGCCCACATTCGCTGAGG-3′ and 5′-CCGGATCCT AATCGCGCAGCTCCATCTTCATAGCCTCGAAGATACCACCCAG CGTGTTGGAGACAGTGGA-3′. Full-length human β_2m was amplified using the primers 5′-GGCATATGATCCAGCGTACTCCAAAG-3′ and 5′-CCGGATCTTACATGTCTCGATCCCA-3′. Polymerase chain reaction products were subcloned into TA vectors (InVitrogen) and then cloned into the NdeI and BamHI sites of the pET3a expression vector (Novagen). Recombinant proteins were generated as insoluble proteins following induction with IPTG in *E. coli* strain BL21 (DE3), and further purified. Insoluble H2-K^d and β_2m were dissolved in 8 M urea and refolded in the presence of 25–100 μg/ml $LLO_{91–99}$, $p60_{217–225}$, $p60_{449–457}$, $mpl_{84–92}$, or the Jak1 self-peptide, and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.4 mM phenylmethylsulfonyl fluoride) to stabilize monomeric H2-K^d/peptide complexes as described (Garboczi et al., 1996). These were purified by gel filtration over a Superdex 200 HR column (Pharmacia) and in vitro biotinylated for 12 hr at 20°C in the presence of 15 μg BirA (AVIDITY, Boulder, CO), 80 μM biotin, 10 mM ATP,10 mM MgOAc, 20 mM bicine, and 10 mM Tris-HCl (pH 8.3). To remove free biotin, monomeric complexes were again purified by gel filtration, tested for efficiency of biotinylation (usually >90%), and then tetramerized by addition of PE–labeled streptavidin (Molecular Probes) at a molar ratio of 4:1. Tetramers were purified by gel filtration using a Superdex 200 HR column and stored at 5 mg/ml at 4°C in phosphate-buffered saline (pH 8.0) containing 0.02% sodium azide, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.5 mM EDTA).

CTL Assays

P815 target cells ($H2^d$) were labeled with ^{51}Cr and washed. FACS-sorted CTL were placed in wells with 10^4 labeled P815 cells in the presence of 10^{-6} M $LLO_{91–99}$, p60–217–225, $p60_{449–457}$, or $mpl_{84–92}$. The release of ^{51}Cr was determined after 4 hr and the percentage of specific lysis calculated as previously described (Pamer et al., 1991).

In Vitro Restimulation of Splenocytes

BALB/c mice were immunized by intravenous infection with 2000 *L. monocytogenes*. 3–4 × 10^7 spleen cells from immunized mice were resuspended in 5 ml $RP10^+$ and added to T 25 cell culture flask. Syngeneic splenocyte stimulators were prepared from naive mice by irradiation (3000 rad) and pulsing for 1 hr at 37°C with 10–8 M $LLO_{91–99}$, 10^{-7} M $p60_{217–225}$, 10^{-6} M $p60_{449–457}$, or 10^{-6} M $mpl_{84–92}$. Cells were washed to remove free, unbound peptide, and 3 × 10^7 cells in 5 ml RP10+ were added to the responder cells. T cell lines were generated by restimulating responders every week with 3 × 10^7 peptide-coated stimulator cells. Following the second restimulation, the medium was supplemented with 5% rat concanavalin A supernatant.

Staining and Analysis of $CD8^+$ T Cells

Splenocytes were enriched for $CD8^+$ T cells using magnetically activated cell sorting (MACS, Miltenyi, Germany) and anti-CD8α microbeads (clone 53–6.7, MACS). For staining, approximately 300,000 $CD8^+$-enriched splenocytes were incubated at 4°C for 20 min with unconjugated streptavidin (0.5 mg/ml, MolecularProbes) and Fc-block (PharMingen), followed by triple staining with CyChr-conjugated anti-CD8α (clone 53–6.7, PharMingen), PE-conjugated tetrameric H2-K^d/peptide complexes (0.2–0.5 mg/ml), and FITC-conjugated anti-CD62L (MEL-14, PharMingen) or FITC-conjugated anti-CD44 (clone IM7.8.1, Caltag) for 1 hr at 4 C. After washing, cells were fixed in 1% paraformaldehyde and then analyzed on a FACScan, using CellQuest software.

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